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Honjoh, Ken-ichi
Laboratory of Food Hygienic Chemistry, Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University

Suga, Koushirou
Laboratory of Food Hygienic Chemistry, Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University

Shinohara, Fuminori
Laboratory of Food Hygienic Chemistry, Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University

Maruyama, Isao
Chlorella Industries Co.Ltd.

他

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Preparation of Protoplasts from *Chlorella vulgaris* K–73122 and Cell Wall Regeneration of Protoplasts from *C. vulgaris* K–73122 and C–27

Ken–ichi HONJOH†, Koushirou SUGA*,, Fuminori SHINOHARA*, Isao MARUYAMA**, Takahisa MIYAMOTO, Shoji HATANO*** and Masayoshi IIO**

Laboratory of Food Hygienic Chemistry, Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812–8581, Japan

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Protoplasts from *Chlorella vulgaris* K–73122 were obtained by enzymatic digestion with a mixture of Acromopeptidase, Cellulase ONOZUKA R–10, Chitosanase KI, Gluczyme, and Uskizyme. The formation of naked protoplasts was confirmed by fluorescence microscopy using fluorescent brightner 28, which stains cell walls. About 88% of *C. vulgaris* K–73122 cells were converted into osmotically–labile cells. Furthermore, a method for regeneration of intact cells from the protoplasts was developed. Utilization of 0.5 M sucrose as an osmoticum, Fe–EDTA as an iron source, and bacto–agar as a supporting was shown to help regeneration of the cell walls of two strains, *C. vulgaris* K–73122 and C–27.

INTRODUCTION

Chlorella has served as one of the model organisms in physiological and biochemical studies of plants for a long time. In applied fields, it has been used in large quantity as health–promoting food for human as well as food for rotifers in cultivation fishery. Recently, *Chlorella* cell components were found to show several bioactivities such as antitumor activity (Noda *et al.*, 1996) and high promotion activity of excretion of dioxins (Morita *et al.*, 2001, 1999). The algae also have been used in biotechnology, for example, for production of useful proteins such as human–growth hormone and so on (Chen *et al.*, 2001; Hawkins and Nakamura, 1999). Some species and strains of *Chlorella* have fast growth rates, which is favorable for industrial mass culture and this could be an additional advantage when the algae are used as materials for various purposes. In many aspects of utilization, however, the great drawback to *Chlorella* cells is that their cell walls are very rigid and are cumbersome barriers against utilization and breeding especially by genetic manipulation such as gene incorporation or cell fusion. Hence, protoplast formation from *Chlorella* cells as well as regeneration of the cell wall to restore the intact cells is a very

* Laboratory of Food Hygienic Chemistry, Division of Food Biotechnology, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812–8581, Japan

** Chlorella Industries Co. Ltd., 1343 Hisatomi, Chikugo, Fukuoka 833–0056, Japan

*** Graduate School of Health and Social Welfare Science, Nishikyushu University, 4490–9 Ooazaozaki, Kanzaki–machi, Kanzaki–gun, Saga 842–8585, Japan

† Corresponding author (E–mail: honjoh@agr.kyushu–u.ac.jp)

important technique to extensively utilize the algae.

So far, many investigations into the chemical composition of cell walls (Blumreisinger *et al.*, 1983; Takeda, 1991, 1988; Takeda and Hirokawa, 1984) and the preparation of protoplasts (Aach *et al.*, 1978; Atkinson *et al.*, 1972; Hatano *et al.*, 1992; Yamada and Sakaguchi, 1981; Yamada *et al.*, 1987) have been carried out with *Chlorella*. However, naked protoplasts from cells of only a few strains have been prepared (Hatano *et al.*, 1992; Yamada and Sakaguchi, 1981; Yamada *et al.*, 1987).

Our previous study showed that osmotically-labile C-27 cells could be produced by treatment of cells with a mixture of chitosanase, mixed glycosidases, and homogenates from C-27 strain after a 3-h digestion (Hatano *et al.*, 1992). Based on the report, we tried to prepare protoplasts of strain K-73122 by enzymatic digestion with several commercially available enzymes. Furthermore, we tried to regenerate protoplast cells to intact cells of the strain and strain C-27.

MATERIALS AND METHODS

Culture of cells

Cells of *Chlorella vulgaris* K-73122 were grown synchronously in Myers-4N medium (Watanabe, 1960), at 25 °C, under a photosynthetic photon flux density of 250 μmol/m^{2}s, with 1.3% CO_2 in air, to a concentration of about 1.0×10^{10} cells per liter, under a 20-h light/4-h dark regime. *C. vulgaris* IAM C-27 was grown synchronously in MC medium under a 16-h light/8-h dark regime and other conditions were the same as those for K-73122 strain. Since cell walls of *C. vulgaris* C-27 were efficiently digested by cell-wall-digesting enzymes at the L_2 stage (an intermediate stage during the ripening phase of the cell cycle; Hatano *et al.*, 1976), cells of *C. vulgaris* K-73122 at the L_2 stage were used for protoplasts preparation.

Preparation of homogenates of *C. vulgaris* K-73122

Homogenates containing the lytic enzymes were prepared from *C. vulgaris* K-73122 as described below. Cells of *C. vulgaris* K-73122 were collected at the L_4 stage (the stage just before cell division; 18 h after the start of the cell cycle) by centrifugation at 2,000×g for 5 min at 4 °C, washed twice with distilled water, and resuspended in distilled water (about 1.5×10^9 cells/ml). The suspension was homogenized with glass beads of 0.5 mm diameter in a reciprocal shaker (Vibrogen-Zellmühle; Edmund Bühler Co., Tübingen, Germany), operated at 4,500 rpm for 20 min at 4 °C. The homogenate was freed from the glass beads by filtration on a sintered-glass funnel and centrifuged at 4,500×g for 20 min at 4 °C. The supernatant was concentrated by lyophilization and used as the *C. vulgaris* K-73122 homogenate.

Preparation of homogenates of rotifer

The rotifer homogenate containing the lytic enzyme was prepared as described below. Frozen rotifer (*Brachionus rotundiformis* obtained from Chlorella Industry Co. Ltd., Tokyo, Japan) was suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF. The suspension was homogenized in a Waring Blender (Dynamics Corp. of America, New Hartford, CT, U.S.A.) for 3 min. The homogenate was centrifuged

at 4,500×g for 20 min at 4 °C. Ammonium sulfate was added to 80% saturation and the mixture was kept in an ice bath for 1 h. After centrifugation, the obtained pellet was dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) with 1 mM PMSF (phenylmethylsulfonylfluoride). The suspension was transferred to dialysis tube (cutoff 3,500: Nacalai Tesque, Kyoto, Japan) and dialyzed at 4 °C against 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF. The dialyzate was used as rotifer homogenate.

Preparation of protoplasts from Chlorella cells

For formation of protoplasts from *C. vulgaris* K–73122, commercially available enzymes were obtained as follows: Acromopeptidase (Ac) and Cellulysine (Cl) were obtained from Wako Pure Chemical Ind. Ltd.; Cellulase ONOZUKA R–10 (Ce) and Macerozyme R–10 (Ma) were obtained from Yakult Pharmaceutical Ind. Co. Ltd.; Chitosanase (Ch), which is derived from *Bacillus* R–4 strain, was obtained K. I. Chemical Industry Co. Ltd.; Gluczyme (Gl) was obtained from Amano Enzyme Inc.; Pectinase (Pe) was obtained from Sigma Chemical Co.; Uskizyme (Us) was obtained from Kyowa Chemical Co.. Each preparation of the enzymes was dissolved in 25 mM sodium phosphate buffer (pH 7.0) that contained 0.5 M mannitol and the final concentrations of the digestive enzymes used were 4.0% (w/v) for Ce and 1.0% (w/v) for the others. When the rotifer homogenate was used by mixing with the other enzymes, 25 mM sodium phosphate buffer (pH 7.0) with 1 M mannitol was used for dissolution of the enzymes. The enzymes were dissolved individually or in combination in the digestion buffer, centrifuged 20,000× g for 20 sec to remove insoluble matters, and then sterilized through polysulfone filters with a 0.45 μm pore size (EB–DISK 25, Kanto Chemical Co., Inc.). Cells were collected at the L_2 stage by centrifugation at 1,000×g for 5 min at room temperature, and then they were resuspended in 25 mM sodium phosphate buffer (pH 7.0) that contained 0.5 M mannitol (about 2.5×10^9 cells/ml). One portion of the suspension was mixed with 8.5 parts (v/v) of the solution of the enzymes, and then the mixture was incubated at 30 °C for 3 h in the dark with shaking at 50 rpm on a reciprocal shaker (model WB–III; TAIYO, Tokyo, Japan). The formation of protoplasts was estimated by addition of 0.1 ml of the suspension to 2.9 ml of distilled water and counting the unburst cells in a Thoma hemacytometer. To more sensitively detect cells with damaged cell walls, 0.1 ml of the suspension was mixed with 2.9 ml of 2% (w/v) sodium dodecyl sulfate (SDS). In the present paper, the osmotically–labile cells and SDS–sensitive cells were defined as spheroplasts and protoplasts, respectively.

Preparation of protoplasts of *C. vulgaris* C–27 was carried out according to the method of Hatano *et al.* (1992).

Fluorescence microscopy

The formation of protoplasts was also revealed by staining the cell wall with fluorescent brightner 28 (FB28; Sigma Chemicals Co.) as described below. *Chlorella* cells, which had been treated with the enzymes, were collected by centrifugation at 800× g for 5 min. The cells were washed three times with 25 mM sodium phosphate buffer (pH 7.0) containing 0.5 M mannitol. Then, the cells were suspended in 0.05% FB 28 in the above buffer and stained under dark for 10 min. After staining, the cells were washed four times with the above buffer, and then observed with a fluorescence microscope (Nikon

Optiphot, dichroic mirror (DM400), excitation: 330 to 380 nm, emission: >420 nm)

Regeneration of cell walls of protoplasts

In order to restore protoplast to intact cells, regeneration of cell walls was tried. Protoplasts obtained from K–73122 and C–27 strains were collected by centrifugation at 800×g for 5 min and washed three times with isotonic buffers, 20 mM Tris–HCl buffer (pH 7.5) containing 0.5 M mannitol, 0.8 M mannitol, 0.4 M sucrose or 0.5 M sucrose to remove the enzymes. Washed protoplasts were resuspended in the same buffer at the concentration of about 10^4 cells/ml. For regeneration of cell walls, PYG medium was used as a basic medium (Table 1). As listed in Table 3, $FeSO_4$ or Fe–EDTA was used as iron source and agar, bacto–agar or agarose was added to PYG medium as the supporting. The protoplast suspension was diluted with the above buffers and the protoplasts were embedded in the regeneration media listed in the Table 4. Regeneration rates were calculated as follows: regeneration rate (%)=(A–B)/(C×factor–B)×100, where factor=D/E, A: colony forming units (cfu) of regeneration cells from protoplasts, B: cfu of osmotically–stable cells treated with sterilized water after digestion with enzymes, C: cfu of cells without digestion with enzymes, D and E: the numbers of enzyme–digested and –undigested cells counted with a Thoma hemacytometer under a microscope, respectively.

Table 1. Composition of PYG medium

Contents	Concentration
Proteose peptone	1.0 (g/l)
Glucose	2.0
Yeast extract	2.0
KNO_3	0.25
K_2HPO_4	0.075
KH_2PO_4	0.175
$MgSO_4 \cdot 7H_2O$	0.075
NaCl	0.025
$CaCl_2 \cdot 7H_2O$	0.01
$FeSO_4 \cdot 7H_2O$ or Fe–EDTA	10.0 or 15.0 (mg/l)
H_3BO_4	2.9
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 7H_2O$	0.08
$(NH_4)_3Mo_7O_{24} \cdot 4H_2O$	0.018
H_2SO_4	0.1 (ml/l)

pH was adjusted to 6.5 with NaOH.

RESULTS AND DISCUSSION

Frequency of protoplasts formation of *Chlorella vulgaris* K–73122

In attempts to prepare protoplasts of *Chlorella*, three characteristics were reported;

it is necessary to keep isotonic buffer concentrations high compared to isotonic buffers for higher plants (Gobel and Aach, 1985); high concentration of cellulase is necessary for preparation of protoplast of *Chlorella*; hemicellulase specifically works well in preparation of protoplast of *Chlorella* though it is not effective in protoplast formation of higher plants (Rosen *et al.*, 1985). Furthermore, rotifer eats *Chlorella* (Hirayama and Nakamura, 1976) and has enzymes, which digest cell walls of *Chlorella*, in its digestive organs (Chun *et al.*, 1997). *Chlorella* itself also has cell-wall-lytic enzymes for division of cells (Hatano *et al.*, 1992). Based on these reports, we started to develop a preparation method of protoplasts of *Chlorella vulgaris* K-73122.

For preparation of protoplasts of *Chlorella vulgaris* K-73122, algal cells at the L_2 stage were treated for 3 h with various combinations of Ac, Ce, Ch, Cl, Gl, Pe, Us, the lytic enzyme in *Chlorella* homogenate, and the lytic enzyme in rotifer homogenate (Table 2).

Table 2. Effects of enzymes on production of SDS-sensitive and osmotically-labile cells of *C. vulgaris* K-73122

Enzyme	Percent of cells	
	SDS-sensitive	Osmotically-labile
K-22 H + rotifer H + Ce + Ch	31	27
rotifer H + Ce + Ch	87	57
K-22 H + Ce + Ch	46	34
K-22 H + rotifer H + Ch	47	17
K-22 H + rotifer H + Ce	36	32
rotifer H + Ce + Ch + Ac	98	72
rotifer H + Ce + Ch + Us	92	68
rotifer H + Ce + Ch + Ac + He	99	72
rotifer H + Ce + Ch + Ac + Us	95	87
rotifer H + Ce + Ch + Ac + Us + Cl	92	45
rotifer H + Ce + Ch + Ac + Us + Gl	91	82
rotifer H + Ce + Ch + Ac + Us + Pe	92	50
Ce + Ch + Ac + Us + Gl	100	88

Cells were treated with the enzymes for 3 h at 30 °C.
K-22 H: K-73122 homogenate.

At first, we tried Ce, Ch, and K-73122 homogenate or rotifer homogenate. For identification of protoplasts and spheroplasts, we counted numbers of osmotically-labile cells and SDS-sensitive cells, respectively. The results showed that the combination of the three enzymes converted only 17% to 57% of the cells into osmotically-labile cells. Especially, K-73122 homogenate did not contribute to spheroplast or protoplast formation, thus, the homogenate was thought to be unnecessary for protoplast formation. Next, the treatment with [the rotifer homogenate, Ce, and Ch] converted 87% of the cells into SDS-sensitive cells. For high efficiency of protoplast formation, effects of addition of the other enzymes to the mixture of [the rotifer homogenate, Ce, and Ch] were investigated. Therefore, we examined Ac, He, and Us as an additional enzyme, one by one. When either Ac or Us was used, the efficiency of protoplast formation was enhanced: the additional use of Ac converted 98% of the cells into SDS-sensitive and 72% of the cells into osmotically-labile. The use of Us converted 92% of the cells into SDS-sensitive and

68% of the cells into osmotically–labile. When only Ac or both Ac and He were added, the efficiencies of protoplast formation were not different, suggesting that He was not necessary for protoplast formation. Furthermore, the additional use of both Ac and Us converted 95% of the cells into SDS–sensitive and 87% of the cells into osmotically–labile. For further good efficiency of protoplast formation, the effects of additional enzymes, Cl, Gl or Pe, were studied. Out of the three enzymes, Gl was the most effective for the formation of protoplasts. On the other hand, whereas the rotifer homogenate occasionally contributed to the formation of protoplasts, the activity of the lytic enzymes in the rotifer homogenate was dependent on the lot of rotifer (data not shown). Hence, we decided not to use the rotifer homogenate. The treatment with [Ce, Ch, Ac, Us, and Gl] converted 100% of the cells into SDS–sensitive and 88% of the cells into osmotically–labile. The method of rapid preparation of protoplasts of *Chlorella vulgaris* K–73122 was established without the rotifer homogenate.

In order to confirm digestion of cell walls of *C. vulgaris* K–73122, fluorescence microscopy was done. The results showed that the number of cells, whose cell walls were stained with fluorescent brightner 28 to light blue, decreased with the treatment time (Fig. 1). After 3 h of the treatment, cell walls stained with fluorescent brightner could not

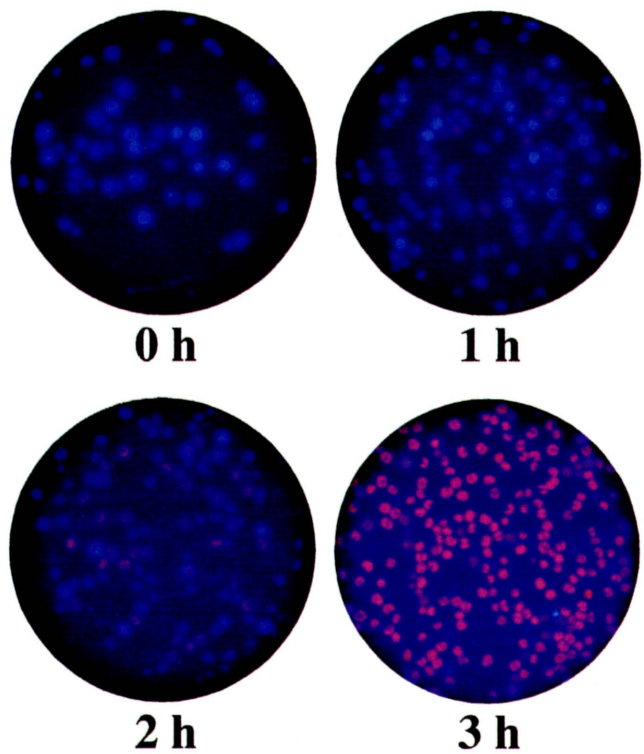

Fig. 1. Fluorescent microphotographs of *C. vulgaris* K–73122 after 0, 1, 2, and 3–h enzyme digestion.

be seen and cells showed red fluorescence based on the chloroplast. The result showed that 3–h treatment of the cells led to almost complete digestion of the cell walls.

The cell–wall composition of *Chlorella* cells dynamically changes during the growth of the cells (Loos and Meindl, 1982). As shown previously (Hatano *et al.*, 1992; Loos and Meindl, 1984), use of endogenous cell–wall–lytic enzymes was thought to be effective for preparation of protoplasts. Although the C–27 homogenate containing the cell–wall–lytic enzymes contributed to the preparation of protoplasts of *C. vulgaris* C–27 (Hatano *et al.*, 1992), K–73122 homogenate did not work in the preparation of protoplasts in the present paper. The homogenates should contain lytic enzymes necessary for cell division. However, the enzymes usually work from inside of the cells and probably digest only some parts of the cell walls. Thus, the homogenate might not work *in vitro* owing to their attack to the cell wall from outside of the cells.

Regeneration rates of cell walls of protoplasts

For regeneration of cell walls of *C. vulgaris* C–27 and K–73122, various conditions were examined. PYG medium was used as a basic medium (Table 1), and effects of supporting media and iron sources on regeneration of cell walls were investigated. Fe–EDTA was reported to be better than $FeSO_4$ as an iron source for growth of *Chlorella* (Myers, 1951; Leone, 1963). Generally, agarose have been used as a supporting medium for growth of higher plants (Shimamoto *et al.*, 1989; Wardrop *et al.*, 1996). In the present paper, we compared three kinds of supporting media (agar, agarose, and bacto–agar). Hence, we investigated the efficiency of combinations of supporting media and iron sources for the regeneration of protoplasts into intact cells. The result showed that Fe–EDTA clearly improved regeneration rates of cell walls of protoplasts of C–27 strain compared to $FeSO_4$ (Table 3). Especially, the combination of bacto–agar and Fe–EDTA led to a good regeneration rate (37.2%). In the case of K–73122 strain, the regeneration rate was also improved although the rate was low (4.2%). So, the combination of Fe–EDTA and bacto–agar was used for further experiments.

Table 3. Regeneration of *C. vulgaris* C–27 and K–73122 protoplasts

Strain	Fe	Agar	Regeneration rate (%)
C. vulgaris C–27	$FeSO_4$	0.4% Agar	6.6
		0.4% Agarose	2.1
		0.6% Bacto–Agar	8.2
	Fe–EDTA	0.4% Agar	29.2
		0.4% Agarose	0
		0.6% Bacto–Agar	37.2
C. vulgaris K–73122	$FeSO_4$	0.4% Agar	0.5
		0.4% Agarose	0
		0.6% Bacto–Agar	0.2
	Fe–EDTA	0.4% Agar	2.4
		0.4% Agarose	0.8
		0.6% Bacto–Agar	4.2

Regeneration rate was calculated as described in Materials and Methods.

Table 4. Effects of mannitol and sucrose in buffers used for preparation and washing of protoplasts of *C. vulgaris* C–27 and K–73122 on regeneration of protoplast cells

Sugar concentration	Regeneration rate (%)	
	Strains	
	C–27	K–73122
0.5 M mannitol	37.2	4.2
0.8 M mannitol	–	13
0.4 M sucrose	27.5	5.1
0.5 M sucrose	40	31.7

Regeneration rate was calculated as described in Materials and Methods.

For better efficiency of regeneration of cell walls of protoplasts, conditions of preparing and washing protoplasts were also studied (Table 4). We examined mannitol and sucrose as an osmoticum, which maintains the osmolarity during preparation of protoplasts and washing cells. In both strains, sucrose was found to work as a good osmoticum. Especially, 31.7% of protoplasts of the strain K–73122 showed colony–forming ability when 0.5 M sucrose was used as an osmoticum. In the case of the strain C–27, using 0.5 M sucrose also improved the regeneration rate up to 40%. Abo–Shady (1990) used PEG1500 as an isotonic stabilizer for regeneration of cell walls of *Chlorella*. We tried to use PEG1500 for regeneration of cell walls of *C. vulgaris* K–73122 and C–27, but the both strains could not form colonies. Thus, sugars, such as sucrose, would be adequate as osmotica for regeneration of cell walls of both strains.

Agars as supporting media are known to contain inorganic matter including calcium salts. Bacto–agar showed best efficiency of regeneration of cell walls and agar showed better efficiency than agarose. This order is conversely proportional to the purification order of agars. For good efficiency of regeneration of cell walls, some impurities seem to be necessary or effective for regeneration of cell walls of protoplasts.

In the case of iron sources, Fe–EDTA showed better efficiency than $FeSO_4$ (Table 3). Under neutral or basic pH, Fe ion tends to form insoluble matter, which are difficult to be transported into cells (Oh–hama and Miyachi, 1988). Thus, because chelating agent EDTA keeps Fe ions soluble, Fe ions could be supplied to protoplasts for effective regeneration of cell walls of *Chlorella*.

Comparison of two types of osmotica, mannitol and sucrose, was done. The results showed that 0.5 M sucrose, compared to mannitol, contributed to much efficient regeneration of cell walls of the protoplasts. Generally, plant cells have facility for incorporation of both sucrose and mannitol (Salmon *et al.*, 1995). However, in *Chlorella* cells, there might be differences in the incorporation rate of mannitol and sucrose. Thus, differences in regulation of osmolarity would affect the regeneration rate of cell walls of the protoplasts.

This report showed the preparation of protoplasts of *Chlorella vulgaris* K–73122 and regeneration of cell walls of *C. vulgaris* strains, K–73122 and C–27. These approaches could underlie genetic modification of *Chlorella* for future technology.

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